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(72)Inventor : ITO KATSU

## (54) HUMAN CELLULAR FIBRONECTIN, ITS PRODUCTION AND CELL STRAIN

(57)Abstract:

PURPOSE: To obtain a human cellular fibronectin useful as a raw material for additives for a medium of animal cell culture, diagnosticums, cosmetics, and medicines, having activity as cell adhesion factor by culturing variant cell strain HUH-6YM derived from human congenital hepatoma and collecting the product from the culture mixture.

CONSTITUTION: Cloning from cell strain HUH-6 clones 5 derived from human congenital hepatoma is repeated, the cell strain is multiplied in a basic medium comprising an amino acid, vitamins, saccharides and inorganic salts, Variant cell strain HUH-6YM derived from human congenital hepatoma which can be limitlessly subjected to subculture without requiring a protein factor as a growth factor and manifests mRNA of fibronectin containing ED-A and ED-B ranges is subjected to subculture at 37° C in air containing 5% CO3 for one month, the cells are inoculated to a medium in a roller bottle, continuously cultured at 37° C under atmospheric pressure for three months, ultrafilterd, concentrated, passed through a gelatin agarose column and purified to give a human cellular fibronectin useful as an additive for a medium in animal cell culture.

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## CLAIMS

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[Claim(s)]
[Claim 1] Human cell nature fibronectin which cell strain HUH-6YM produces.
[Claim 2] The manufacture approach of the human cell nature fibronectin characterized by cultivating cell strain HUH-6YM and separating human cell nature fibronectin from the obtained culture.
[Claim 3] Cell strain HUH-6YM which has the following property.
** It is the variant of the Homo sapiens hepatoblastoma origin cell strain HUH-6clone5 origin.
** Increase by the basal medium which consists of amino acid, vitamins, a saccharide, and mineral, and subculture is possible without a limitation. That is, it is the cell strain which does not need a protein sex factor as a growth factor.
** HUH-6YM carries out remarkable production of the human cell nature fibronectin.
** mRNA of fibronectin including ED-A and an ED-B field is discovered.

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[Translation done.]

## DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to cell strain HUH-6YM of the Homo sapiens hepatoblastoma origin which uses human cell nature fibronectin, its manufacturing method, and this approach. Fibronectin is useful as an additive of the culture medium in animal cell culture, and has an application as a diagnostic drug, cosmetics, and a drugs raw material further.
[0002]
[Description of the Prior Art] Generally, fibronectin is bioactive protein which has the operation as a cell adhesion sex factor. Two kinds of cellularity fibronectins which fibroblast mainly produces as the class with the plasma nature fibronectin which exists in plasma, and exist during an organization are known. It is reported that the operation of such fibronectins is participating not only in adhesion and an expansion operation of a cell but in phenomena, such as the chemotaxis of a cell, phagocytosis, differentiation, cell morphallaxis, cell movement, gun-izing, and gun transition, deeply. Since fibronectin has various operations to the cell in this way, application to drugs like the remedy to tissue damage and application to the cosmetics which make the condition of skin tissue good are advanced. Moreover, application to the diagnostic drug using the property specifically combined with various matter, such as heparin of fibronectin and a collagen, and application as a diagnostic drug marker are also advanced.
[0003] However, fibronectin is the factor of the protein nature originating in an animal tissue or its plasma, and in order to prepare in large quantities, it has problems, such as mixing of the problem of reservation of the raw material and the impurity of the raw material origin to the refined material, for example, a virus etc.
[0004] Although the approach of extracting from a Homo sapiens placenta or refining from the culture supernatant of fibroblast was learned in order to obtain human cell nature fibronectin furthermore, it was difficult to obtain only the cellularity fibronectin of a minute amount but to prepare all in large quantities.
[0005]
[Problem(s) to be Solved by the Invention] This invention aims at preparing human cell nature fibronectin in large quantities.
[0006]
[Means for Solving the Problem] this invention persons could cultivate by the serum free medium to which HUH-6clone5 (Japanese Cancer Research Resources Bank, cell number JCRB0401) previously separated from a Homo sapiens hepatoblastoma added an insulin independent as a protein sex factor, it found out producing remarkable plasma nature fibronectin in the culture supernatant, and patent application of the manufacturing method of the plasma nature fibronectin using it was carried out. (JP,3-160989,A) .
[0007] Then, when this invention persons inquired by repeating cloning from HUH-6clone5 further,

they found out Homo sapiens hepatoblastoma origin variant HUH-6YM (hereafter referred to as cell strain HUH-6YM.) which can carry out subculture without a limitation in a protein additive-free culture medium that it can cultivate, and established this as an established cell line.
[0008] This established cell line is FERM as cell strain HUH-6YM. It ****s to Fermentation Research Institute by P-12890.
[0009] Furthermore, for the unexpected thing, the fibronectin which this cell strain HUH-6YM produces became clear [ that it is cellularity fibronectin ], and this cellularity fibronectin found out that it was high solubility to the cane-sugar solution. This invention is completed based on the above-mentioned knowledge, and it cultivates the human cell nature fibronectin which cell strain HUH-6YM produces, and cell strain HUH-6YM, separates and refines cellularity fibronectin from the obtained culture medium, and is related with cell strain HUH-6YM which is the new Homo sapiens hepatoblastoma origin cell strain used for the manufacture approach and approach of the cellularity fibronectin characterized by preparing to high concentration.
[0010]
[Effect of the Invention] By this invention, the method of manufacturing human cell nature fibronectin in large quantities was offered. Since the cellularity fibronectin obtained by this manufacture approach can obtain cell strain HUH-6YM from the supernatant liquid cultivated by the protein additive-free culture medium, it does not have the risk of mixing, such as different-species protein and a virus.
[0011] Moreover, it was shown clearly as a result of this invention that human cell nature fibronectin could be mass-produced using the cell strain obtained by selection and the transformation of a cell.
[0012] Furthermore, cellularity fibronectin is especially considered to be an important factor about migration and chemotaxis of a cell from having discovered more acting, in case fibronectin is implantation of a fertilized egg, and cellularity (Wartiovaara, J.etal., Develop.Biol., 1979; 247-257) fibronectins in an embryo organization from the adult organization unlike plasma nature fibronectin (28 Oyama, F.etal., Biochemistry, 1989; 1428) etc.
[0013] That is, the use as drugs characteristic as a factor which maintains the homeostasis of a **** repairing agent or an epidermal cell for the cell migration at the time of tissue damage, cosmetics, and a diagnostic drug is possible. Moreover, since it has the same biological activity as plasma nature fibronectin fundamentally, it can use for the same application.
[0014]
[Detailed Description of the Invention] Cell strain HUH-6YM used for this invention is the variant obtained in HUH-6clone5 as a result of carrying out cloning on cell level, it is clearly [ HUH-6clone5 ] different, and a biological variation is seen. Although the chromosome number of already reported HUH-6clone5 is 49 (Sato, J.et al;Acta Med.Okayama34 (2), 127-130 (1980)), it is specific at the point that there can be more chromosome numbers of HUH-6YM than it, and cell strain HUH-6YM can be increased by the protein additive-free culture medium.
[0015] This cell strain HUH-6YM was specifically obtained by the croning process shown below. The phase diluent of HUH-6clone5 was produced using the e-RDF culture medium (583 Japan Society for Bioscience, Biotechnology and Agrochemistry Vol. 58, p575- 1984) which contains an insulin (0.04 IU/ml) first, seeding was carried out to the petri dish with a diameter of 100mm, and it cultivated for four weeks from two weeks under 5%CO2 content air and 37-degree-C conditions.
[0016] The colony considered to have been formed from the single cell among the formed colonies was able to be chosen, and it was able to plant in 24 hole plate, and was able to inherit, several cloning was able to be performed for the clone with many volumes of cellularity fibronectin using the same approach as the point in it, and cell strain HUH-6YM was able to be obtained. Moreover, at least the e-RDF culture medium of this cell strain HUH-6YM was possible for growth and a passage.

[0017] Moreover, when the cDNA probe to the peptide called ED-A and the ED-B field which exist only in cellularity fibronectin was produced and mRNA of HUH-6YM was investigated, existence of

ED-A and an ED-B field was checked.
[0018] Thus, it is as follows when the biological property of obtained cell strain HUH-6YM is summarized.
** It is the variant of the Homo sapiens hepatoblastoma origin cell strain HUH-6clone5 origin.
** Increase by the basal medium which consists of amino acid, vitamins, a saccharide, and mineral, and subculture is possible without a limitation. That is, it is the cell strain which does not need a protein sex factor as a growth factor.
** Carry out remarkable production of the human cell nature fibronectin.
** mRNA of fibronectin including ED-A and an ED-B field is discovered.
[0019] Next, cell strain HUH-6YM is cultivated and how to manufacture human cell nature fibronectin is explained.
[0020] Culture of cell strain HUH-6YM is cultivated with a petri dish as seed culture. A passage is repeated and the required number of cells is secured by the next continuous culture. Although the e-RDF culture medium marketed is suitable as a culture medium to be used, anythings of the culture medium which is not limited to this and prepared according to the component are usable. Moreover, as an addition component, although especially a protein sex factor is unnecessary, unless it has a bad influence on a cell, it may be added.
[0021] Although especially a culture condition is not specified, culture temperature is 36-37 degrees C, and is suitable for gaseous-phase conditions. [ of the air which contains about 5% of CO2 in seed culture with a petri dish etc. ] However, there is no need for air of having prepared CO2 grade especially in continuous culture.
[0022] Although especially the approach of continuous culture is not limited, it can be carried out by the approach using the roller bottle which is a general approach. In this case, according to the number of cells in that roller bottle, culture media are exchanged at the suitable stage.
[0023] The culture supernatant of cell strain HUH-6YM obtained by such approach serves as a raw material of the next purification.
[0024] Although the approach used for general protein purification can be used for purification of cellularity fibronectin, it will be as follows if one of them is raised.
[0025] After adding the suitable protease inhibitor for a culture supernatant first, then giving centrifugal separation and removing impurities, such as a piece of a cell, it condenses by the extra filtrating method and the cellularity fibronectin in a culture supernatant is raised even to suitable concentration. This is for increasing the efficiency of the next purification. The load of the condensed supernatant liquid is carried out to the gelatin affinity column which equilibrated by PBS (-) and (Phosphate Buffered Saline). Then, elution is carried out with the buffer solution which dissolved 6M urea in PBS (-), and the elution fraction is obtained. In order for the purity of the cellularity fibronectin in the inside of this fraction to be 95% or more according to assay by the electrophoresis method, but to raise purity further, it can high-grade-ize using other approaches, such as a general ion-exchange method, but in the buffer solution usually like PBS (-), since solubility is very low, cellularity fibronectin needs to perform separation actuation under urea existence altogether.
[0026] Then, a urea is removed, and in order to prepare the cellularity fibronectin solution more than a certain concentration, the phosphate buffer solution and buffer-solution exchange which contain cane sugar 5% are performed. It can carry out by the diafiltration method using the film dialysis and extra filtration which are a general approach as an approach of exchanging with the buffer solution.
[0027] Thus, the human cell nature fibronectin obtained is obtained as mixture of a dimer and a monomer. As a description on the structure of cellularity fibronectin, according to analysis of the SDS polyacrylamide gel migration under a reduction condition and nonreduction conditions, the molecular weight is larger than a plasma nature fibronectin preparation, and it reacts with a commercial anti-cellularity fibronectin antibody.
[0028] Many properties of the human cell nature fibronectin which used and manufactured cell

strain HUH-6YM below are shown.
** By the SDS polyacrylamide gel electrophoresis in molecular weight reduction and a nonreduction condition, a dimer shows 470kd(s), a monomer shows 245kd(s) and 225kd(s), and the ratio of a dimer and a monomer is 2:1.
** Isoelectric point : react with the anti-cellularity fibronectin antibody (sigma company make, MONOCLONAL ANTI-CELLULAR FIBRONECTIN, Product No.F6140) of pH5 - 6** immunologic specificity marketing, and an anti-Homo sapiens fibronectin antibody (the TAKARA SHUZO make, clone 30-8) reacts.
** The fragments by the protease of the human cell nature fibronectin of this invention differed as clearly as the protease fragment of human plasma nature fibronectin as a result of the fragmentation by the protease.
Moreover, the human cell nature fibronectin manufactured by this invention has a biological operation of fibronectins, such as cell adhesion promotion activity, gelatin bead condensation ability, cell expansion activity, etc. which have already been reported.
[0029] Human cell nature fibronectin can be manufactured very advantageous using cell strain HUH-6YM by carrying out continuous culture of a lot of cells by the protein additive-free culture medium as mentioned above.
[0030]
[Example]
[Production of I. matter] Subculture of the cell strain HUH-6YM (FERM P-12890) was carried out using an e-RDF culture medium (product made from Far East Pharmaceuticals) as seed culture. Culture is 37 degrees C and the air which contains CO2 5%, and was performed for one month.
[0031] The continuous culture for obtaining a culture supernatant carried out seeding of the 1x109 cells per 1750cm roller bottle (falcon company make) of 2, and cultivated them under 37 degrees C and atmospheric air. After seeding, at intervals of two - three days, whole-quantity exchange of the 500ml [ per roller bottle ] culture medium was carried out after that every day, and the obtained culture supernatants were collected till three weeks. Continuous culture was performed for about three months. ( Drawing 1 )
[0032] After the culture supernatant added 5mM ethylenediaminetetraacetic acid sodium (EDTA) as protease inhibitor, continuation centrifugal separation of it was carried out, it removed impurities, such as a cell fragment, by part for 1500g and rate-of-flow 20L/, and (H600made from domestic-S), and collected supernatants.
[0033] Next, the 10 time concentration liquid of a previous supernatant was prepared using the ultrafiltration film of cut-off-molecular-weight 100k (fill TRON company make, form OS100CO5). The gelatin sepharose 4B column (Pharmacia manufacture) load of this was equilibrated and carried out by PBS (-), and it washed and equilibrated by PBS (-). Then, the buffer solution made to dissolve 6M urea in PBS (-) performed elution.
[0034] The purity of the cellularity fibronectin in this eluate was 95% or more as a result of assay by the SDS-polyacrylamide-gel-electrophoresis method under reduced condition.
[0035] Using the ultrafiltration film (fill TRON company make, form OS-100CO1) of cut-off-molecular-weight 100k, the eluate was condensed first, these eluates were collected and the 0.1M sodium phosphate buffer solution and solvent exchange which contain cane sugar 5% were continuously performed by the diafiltration method.
[0036] The human cell nature fibronectin preparation was obtained by the above approaches.
[0037]
[The property of II. matter]
(1) Molecular weight [0038] The molecular weight of the above and a human cell nature fibronectin preparation was measured by the SDS-polyacrylamide-gel-electrophoresis method under standard protein and a human plasma nature fibronectin preparation (Iwaki Glass Co., Ltd. make), reduction, and a nonreduction condition.

[0039] Consequently, the dimer was presumed to be 470kd(s), the monomers were presumed to be 245kd(s) and 225kd(s), and the molecular weight of the human cell nature fibronectin preparation of this invention did not have clearly having bigger molecular weight than human plasma nature fibronectin. ( Drawing 2 )
(2) immunological reactivity -- [0040] The reactant check was performed by the immunity staining technique after Western blotting using the anti-cellularity fibronectin (sigma company make, Product No.F-6140) which does not react to plasma nature fibronectin but reacts only to cellularity fibronectin. Consequently, this preparation reacted with this specific antibody. Moreover, the Homo sapiens fibronectin antibody (the TAKARA SHUZO make, clone 30-8) reacted.
(3) Fragmentation by the protease [0041] The difference of the molecular weight of the fragment by the protease of human plasma nature fibronectin and the cellularity fibronectin of this invention was examined using thermolysin. The immunological reactivity of the fragment object furthermore obtained was examined.
[0042] Consequently, as for human plasma nature fibronectin, the magnitude of the fragment by the protease differed clearly, and the cellularity fibronectin of this invention was considered to be the cellularity fibronectin which includes ED-A and an ED-B field with the immunological reactivity of the fragment by the monoclonal antibody of a pan.
[0043] It was judged as a result of analysis of mRNA of the fragment by the above-mentioned molecular weight, immunological reactivity, and the protease, and a cell that the fibronectin obtained by this invention was human cell nature fibronectin including ED-A and an ED-B field.
[0044] (4) According to the water-soluble previous report, it is reported that cellularity fibronectin has very low solubility from pH6 to the buffer solution of 8.
[0045] In this invention, it was found out to the buffer solution with which cellularity fibronectin contains cane sugar that it is soluble. The solubility over each cane-sugar concentration is shown in the next table 1.
[0046]
[Table 1]

表１．ショ糖溶液に対する溶解性

| ショ糖濃度 | 透析後$OD_{280}$ | 初期液量 | 最終液量 | 回収率 |
|---|---|---|---|---|
| 0％ | 1.37 | 3 mL | 3.0mL | 66％ |
| 1％ | 1.53 | 4 mL | 3.3mL | 60％ |
| 3％ | 1.93 | 3 mL | 2.5mL | 77％ |
| 5％ | 2.45 | 3 mL | 2.3mL | 90％ |
| 10％ | 3.78 | 3 mL | 1.5mL | 90％ |

回収率＝(初期液量×透析後$OD_{280}$／1.3*)／(最終液量×2.08**
(％)　／1.3) ×100
* 細胞性フィブロネクチン濃度１mg／mlの時、$OD_{280}$＝1.3
** 透析前サンプル溶液の$OD_{280}$

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* NOTICES *



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PRIOR ART

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[Description of the Prior Art] Generally, fibronectin is bioactive protein which has the operation as a cell adhesion sex factor. Two kinds of cellularity fibronectins which fibroblast mainly produces as the class with the plasma nature fibronectin which exists in plasma, and exist during an organization are known. It is reported that the operation of such fibronectins is participating not only in adhesion and an expansion operation of a cell but in phenomena, such as the chemotaxis of a cell, phagocytosis, differentiation, cell morphallaxis, cell movement, gun-izing, and gun transition, deeply. Since fibronectin has various operations to the cell in this way, application to drugs like the remedy to tissue damage and application to the cosmetics which make the condition of skin tissue good are advanced. Moreover, application to the diagnostic drug using the property specifically combined with various matter, such as heparin of fibronectin and a collagen, and application as a diagnostic drug marker are also advanced.
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## EFFECT OF THE INVENTION

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[0011] Moreover, it was shown clearly as a result of this invention that human cell nature fibronectin could be mass-produced using the cell strain obtained by selection and the transformation of a cell.
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[0026] Then, a urea is removed, and in order to prepare the cellularity fibronectin solution more than a certain concentration, the phosphate buffer solution and buffer-solution exchange which contain cane sugar 5% are performed. It can carry out by the diafiltration method using the film dialysis and extra filtration which are a general approach as an approach of exchanging with the buffer solution.
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[0029] Human cell nature fibronectin can be manufactured very advantageous using cell strain HUH-6YM by carrying out continuous culture of a lot of cells by the protein additive-free culture medium as mentioned above.

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## TECHNICAL PROBLEM

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[Problem(s) to be Solved by the Invention] This invention aims at preparing human cell nature fibronectin in large quantities.

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[Translation done.]

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MEANS

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[Means for Solving the Problem] this invention persons could cultivate by the serum free medium to which HUH-6clone5 (Japanese Cancer Research Resources Bank, cell number JCRB0401) previously separated from a Homo sapiens hepatoblastoma added an insulin independent as a protein sex factor, it found out producing remarkable plasma nature fibronectin in the culture supernatant, and patent application of the manufacturing method of the plasma nature fibronectin using it was carried out. (JP,3-160989,A) .
[0007] Then, when this invention persons inquired by repeating cloning from HUH-6clone5 further, they found out Homo sapiens hepatoblastoma origin variant HUH-6YM (hereafter referred to as cell strain HUH-6YM.) which can carry out subculture without a limitation in a protein additive-free culture medium that it can cultivate, and established this as an established cell line.
[0008] This established cell line is FERM as cell strain HUH-6YM. It ****s to Fermentation Research Institute by P-12890.
[0009] Furthermore, for the unexpected thing, the fibronectin which this cell strain HUH-6YM produces became clear [ that it is cellularity fibronectin ], and this cellularity fibronectin found out that it was high solubility to the cane-sugar solution. This invention is completed based on the above-mentioned knowledge, and it cultivates the human cell nature fibronectin which cell strain HUH-6YM produces, and cell strain HUH-6YM, separates and refines cellularity fibronectin from the obtained culture medium, and is related with cell strain HUH-6YM which is the new Homo sapiens hepatoblastoma origin cell strain used for the manufacture approach and approach of the cellularity fibronectin characterized by preparing to high concentration.

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[Translation done.]

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EXAMPLE

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[Example]
[Production of I. matter] Subculture of the cell strain HUH-6YM (FERM P-12890) was carried out using an e-RDF culture medium (product made from Far East Pharmaceuticals) as seed culture. Culture is 37 degrees C and the air which contains CO2 5%, and was performed for one month.
[0031] The continuous culture for obtaining a culture supernatant carried out seeding of the 1x109 cells per 1750cm roller bottle (falcon company make) of 2, and cultivated them under 37 degrees C and atmospheric air. After seeding, at intervals of two - three days, whole-quantity exchange of the 500ml [ per roller bottle ] culture medium was carried out after that every day, and the obtained culture supernatants were collected till three weeks. Continuous culture was performed for about three months. ( Drawing 1 )
[0032] After the culture supernatant added 5mM ethylenediaminetetraacetic acid sodium (EDTA) as protease inhibitor, continuation centrifugal separation of it was carried out, it removed impurities, such as a cell fragment, by part for 1500g and rate-of-flow 20L/, and (H600made from domestic-S), and collected supernatants.
[0033] Next, the 10 time concentration liquid of a previous supernatant was prepared using the ultrafiltration film of cut-off-molecular-weight 100k (fill TRON company make, form OS100CO5). The gelatin sepharose 4B column (Pharmacia manufacture) load of this was equilibrated and carried out by PBS (-), and it washed and equilibrated by PBS (-). Then, the buffer solution made to dissolve 6M urea in PBS (-) performed elution.
[0034] The purity of the cellularity fibronectin in this eluate was 95% or more as a result of assay by the SDS-polyacrylamide-gel-electrophoresis method under reduced condition.
[0035] Using the ultrafiltration film (fill TRON company make, form OS-100CO1) of cut-off-molecular-weight 100k, the eluate was condensed first, these eluates were collected and the 0.1M sodium phosphate buffer solution and solvent exchange which contain cane sugar 5% were continuously performed by the diafiltration method.
[0036] The human cell nature fibronectin preparation was obtained by the above approaches.
[0037]
[The property of II. matter]
(1) Molecular weight [0038] The molecular weight of the above and a human cell nature fibronectin preparation was measured by the SDS-polyacrylamide-gel-electrophoresis method under standard protein and a human plasma nature fibronectin preparation (Iwaki Glass Co., Ltd. make), reduction, and a nonreduction condition.
[0039] Consequently, the dimer was presumed to be 470kd(s), the monomers were presumed to be 245kd(s) and 225kd(s), and the molecular weight of the human cell nature fibronectin preparation of this invention did not have clearly having bigger molecular weight than human plasma nature fibronectin. ( Drawing 2 )
(2) immunological reactivity — [0040] The reactant check was performed by the immunity staining

technique after Western blotting using the anti-cellularity fibronectin (sigma company make, Product No.F-6140) which does not react to plasma nature fibronectin but reacts only to cellularity fibronectin. Consequently, this preparation reacted with this specific antibody. Moreover, the Homo sapiens fibronectin antibody (the TAKARA SHUZO make, clone 30-8) reacted.
(3) Fragmentation by the protease [0041] The difference of the molecular weight of the fragment by the protease of human plasma nature fibronectin and the cellularity fibronectin of this invention was examined using thermolysin. The immunological reactivity of the fragment object furthermore obtained was examined.
[0042] Consequently, as for human plasma nature fibronectin, the magnitude of the fragment by the protease differed clearly, and the cellularity fibronectin of this invention was considered to be the cellularity fibronectin which includes ED-A and an ED-B field with the immunological reactivity of the fragment by the monoclonal antibody of a pan.
[0043] It was judged as a result of analysis of mRNA of the fragment by the above-mentioned molecular weight, immunological reactivity, and the protease, and a cell that the fibronectin obtained by this invention was human cell nature fibronectin including ED-A and an ED-B field.
[0044] (4) According to the water-soluble previous report, it is reported that cellularity fibronectin has very low solubility from pH6 to the buffer solution of 8.
[0045] In this invention, it was found out to the buffer solution with which cellularity fibronectin contains cane sugar that it is soluble. The solubility over each cane-sugar concentration is shown in the next table 1.
[0046]
[Table 1]

表１．ショ糖溶液に対する溶解性

| ショ糖濃度 | 透析後$OD_{280}$ | 初期液量 | 最終液量 | 回収率 |
|---|---|---|---|---|
| ０％ | 1.37 | ３mL | 3.0mL | 66％ |
| １％ | 1.53 | ４mL | 3.3mL | 60％ |
| ３％ | 1.93 | ３mL | 2.5mL | 77％ |
| ５％ | 2.45 | ３mL | 2.3mL | 90％ |
| 10％ | 3.78 | ３mL | 1.5mL | 90％ |

回収率＝(初期液量×透析後$OD_{280}$／1.3*)／(最終液量×2.08**
(％)　／1.3) ×100

* 細胞性フィブロネクチン濃度１mg／mlの時、$OD_{280}$＝1.3

** 透析前サンプル溶液の$OD_{280}$

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[Translation done.]

* NOTICES *



1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

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## DESCRIPTION OF DRAWINGS

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[Brief Description of the Drawings]
[Drawing 1] Drawing showing the change to the incubation period of the fibronectin concentration which cell strain HUH-6YM produces in a culture supernatant during continuous culture.
[Drawing 2] The result of having applied the human cell nature fibronectin of this invention to SDS polyacrylamide gel electrophoresis is shown. The human plasma nature fibronectin preparation is used as comparison contrast.

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[Translation done.]

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(54)【発明の名称】　ヒト細胞性フィブロネクチン、その製造法及び細胞株

(57)【要約】　（修正有）
【構成】　細胞株ＨＵＨ－６ＹＭの生産するヒト細胞性フィブロネクチンとその製造方法。細胞株ＨＵＨ－６ＹＭは下記の性質を有する。
①ヒト肝芽腫由来細胞株ＨＵＨ－６ｃｌｏｎｅ５由来の変異株である。
②アミノ酸、ビタミン類、糖類及び無機塩類から成る基礎培地で増殖し、限界なく継代培養が可能である。すなわち増殖因子としてタンパク性因子を必要としない細胞株である。
③ＨＵＨ－６ＹＭは、ヒト細胞性フィブロネクチンを著量産生する。
④ＥＤ－Ａ及びＥＤ－Ｂ領域を含むフィブロネクチンのｍＲＮＡが発現されている。
【効果】　動物細胞培養における培地の添加剤として有用である。

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【特許請求の範囲】
【請求項１】　細胞株ＨＵＨ－６ＹＭの生産するヒト細胞性フィブロネクチン。
【請求項２】　細胞株ＨＵＨ－６ＹＭを培養し、得られた培養物からヒト細胞性フィブロネクチンを分離することを特徴とするヒト細胞性フィブロネクチンの製造方法。
【請求項３】　下記の性質を有する細胞株ＨＵＨ－６ＹＭ。
①ヒト肝芽腫由来細胞株ＨＵＨ－６ｃｌｏｎｅ５由来の変異株である。
②アミノ酸、ビタミン類、糖類及び無機塩類から成る基礎培地で増殖し、限界なく継代培養が可能である。すなわち増殖因子としてタンパク性因子を必要としない細胞株である。
③ＨＵＨ－６ＹＭは、ヒト細胞性フィブロネクチンを著量産生する。
④ＥＤ－Ａ及びＥＤ－Ｂ領域を含むフィブロネクチンのｍＲＮＡが発現されている。
【発明の詳細な説明】
【０００１】
【産業上の利用分野】本発明は、ヒト細胞性フィブロネクチンとその製造法及びこの方法を用いるヒト肝芽腫由来の細胞株ＨＵＨ－６ＹＭに関する。フィブロネクチンは、動物細胞培養における培地の添加剤として有用であり、さらに診断薬、化粧品及び医薬品原料としての用途を有する。
【０００２】
【従来の技術】一般に、フィブロネクチンは、細胞接着性因子としての作用を有する生理活性タンパク質である。その種類としては、血漿中に存在する血漿性フィブロネクチンと主に線維芽細胞が産生し組織中に存在する細胞性フィブロネクチンの２種類が知られている。これらのフィブロネクチンの作用は、細胞の接着及び伸展作用だけではなく、細胞の走化性、食作用、分化、細胞形態調節、細胞運動、ガン化及びガン転移等の現象にも深く関与していることが報告されている。フィブロネクチンは、このように細胞に対しさまざまな作用を有していることから、組織損傷に対する治療薬のような医薬品への応用や、皮膚組織の状態を良好にする化粧品への応用が進められている。また、フィブロネクチンのヘパリンやコラーゲン等のさまざまな物質に特異的に結合する性質を利用した診断薬への応用や、診断薬マーカーとしての応用も進められている。
【０００３】しかしながら、フィブロネクチンは動物組織又はその血漿等に由来するタンパク性の因子で、大量に調製するためには、その原料の確保の問題及びその精製品に対する原料由来の不純物、例えば、ウイルス等の混入などの問題がある。
【０００４】さらにヒト細胞性フィブロネクチンを得るためには、ヒト胎盤から抽出するか線維芽細胞の培養上清から精製する方法が知られていたが、いずれも微量の細胞性フィブロネクチンしか得られず、大量に調製することは困難であった。

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【０００５】
【発明が解決しようとする課題】本発明はヒト細胞性フィブロネクチンを大量に調製することを目的としている。
【０００６】
【課題を解決するための手段】本発明者らは、先にヒト肝芽腫から分離されたＨＵＨ－６ｃｌｏｎｅ５（Ｊａｐａｎｅｓｅ　Ｃａｎｃｅｒ　Ｒｅｓｅａｒｃｈ　Ｒｅｓｏｕｒｃｅｓ　Ｂａｎｋ，細胞番号ＪＣＲＢ０４０１）がタンパク性因子としてインシュリン単独を添加した無血清培地で培養可能で、その培養上清中に著量の血漿性フィブロネクチンを産生していることを見いだし、それを用いた血漿性フィブロネクチンの製造法を特許出願した。（特開平３－１６０９８９）。
【０００７】そこで本発明者らは、更にＨＵＨ－６ｃｌｏｎｅ５からクローニングを繰り返し、検討を行ったところ、タンパク質無添加培地で培養が可能で、且つ限界なく継代培養できるヒト肝芽腫由来変異株ＨＵＨ－６ＹＭ（以下、細胞株ＨＵＨ－６ＹＭとする。）を見いだし、これを株化細胞として確立した。
【０００８】本株化細胞は細胞株ＨＵＨ－６ＹＭとして、ＦＥＲＭ　Ｐ－１２８９０で微工研に寄託されている。
【０００９】更に、意外なことには、本細胞株ＨＵＨ－６ＹＭが産生するフィブロネクチンは、細胞性フィブロネクチンであることが明らかとなり、またこの細胞性フィブロネクチンがショ糖溶液に対し高溶解性であることを見いだした。本発明は、上記知見に基づき完成されたものであり、それは細胞株ＨＵＨ－６ＹＭの生産するヒト細胞性フィブロネクチン及び細胞株ＨＵＨ－６ＹＭを培養し、得られた培養液から細胞性フィブロネクチンを分離、精製し、高濃度に調製することを特徴とする細胞性フィブロネクチンの製造方法とその方法に用いる新規なヒト肝芽腫由来細胞株である細胞株ＨＵＨ－６ＹＭに関するものである。
【００１０】
【発明の効果】本発明により、ヒト細胞性フィブロネクチンを大量に製造する方法が提供された。本製造方法によって得られる細胞性フィブロネクチンは、細胞株ＨＵＨ－６ＹＭをタンパク質無添加培地で培養した上清から得ることができるので、異種タンパク質やウイルス等の混入の危険がない。
【００１１】また本発明の結果、ヒト細胞性フィブロネクチンは、細胞の選択及び形質転換によって得られた細胞株を用いて大量生産が可能であることが明らかにされた。

【００１２】さらに、フィブロネクチンが受精卵の着床の際に作用することや、（Ｗａｒｔｉｏｖａａｒａ，Ｊ．ｅｔａｌ．，Ｄｅｖｅｌｏｐ．Ｂｉｏｌ．，１９７９；２４７－２５７）細胞性フィブロネクチンは血漿性フィブロネクチンとは異なり成人組織より胎児組織においてより多く発現していること（Ｏｙａｍａ，Ｆ．ｅｔａｌ．，Ｂｉｏｃｈｅｍｉｓｔｒｙ，１９８９；２８，１４２８）などから、特に細胞性フィブロネクチンは、細胞の移動及び走化性に関して重要な因子であると考えられる。

【００１３】つまり組織損傷時の細胞移動を供う修復剤、あるいは表皮細胞の恒常性を保つ因子などとして特徴的な医薬品、化粧品及び診断薬としての利用が可能である。また基本的には血漿性フィブロネクチンと同様な生物学的活性を有する為、同様な用途に利用できる。

【００１４】

【発明の具体的な説明】本発明に用いられる細胞株ＨＵＨ－６ＹＭは、ＨＵＨ－６ｃｌｏｎｅ５を細胞レベルでクローニングをした結果得られた変異株であり、ＨＵＨ－６ｃｌｏｎｅ５とは明らかに相違し、生物学的な変異が見られる。既に報告されているＨＵＨ－６ｃｌｏｎｅ５の染色体数は４９本であるが（Ｓａｔｏ，Ｊ．ｅｔａｌ；Ａｃｔａ　Ｍｅｄ．Ｏｋａｙａｍａ３４（２），１２７－１３０（１９８０））、ＨＵＨ－６ＹＭの染色体数はそれより多く、また細胞株ＨＵＨ－６ＹＭはタンパク質無添加培地で増殖可能である点で特異的である。

【００１５】この細胞株ＨＵＨ－６ＹＭは、具体的には以下に示すクローニング法により得られた。まずインシュリン（０．０４ＩＵ／ｍｌ）を含むｅ－ＲＤＦ培地（日本農芸化学会誌Ｖｏｌ．５８，ｐ５７５－５８３，１９８４）を用いてＨＵＨ－６ｃｌｏｎｅ５の段階希釈液を作製し、直径１００ｍｍのシャーレに播種し、５％$CO_2$含有空気、３７℃条件下での２週間から４週間培養した。

【００１６】形成したコロニーのうち、単一細胞から形成されたと考えられるコロニーを選択し、２４穴プレートに植え継ぎ、その中で細胞性フィブロネクチンの生産量の多いクローンを先と同様な方法を用いて数次クローニングを行い、細胞株ＨＵＨ－６ＹＭを得ることができた。またこの細胞株ＨＵＨ－６ＹＭはｅ－ＲＤＦ培地だけでも増殖及び継代が可能であった。

【００１７】また、細胞性フィブロネクチンのみに存在するＥＤ－Ａ及びＥＤ－Ｂ領域と呼ばれるペプチドに対するｃＤＮＡプローブを作製し、ＨＵＨ－６ＹＭのｍＲＮＡを調べたところ、ＥＤ－Ａ及びＥＤ－Ｂ領域の存在が確認された。

【００１８】このようにして得られた細胞株ＨＵＨ－６ＹＭの生物学的性質をまとめると以下の通りである。

①ヒト肝芽腫由来細胞株ＨＵＨ－６ｃｌｏｎｅ５由来の変異株である。



②アミノ酸、ビタミン類、糖類及び無機塩類から成る基礎培地で増殖し、限界なく継代培養が可能である。すなわち増殖因子としてタンパク性因子を必要としない細胞株である。

③ヒト細胞性フィブロネクチンを著量産生する。

④ＥＤ－Ａ及びＥＤ－Ｂ領域を含むフィブロネクチンのｍＲＮＡが発現されている。

【００１９】次に細胞株ＨＵＨ－６ＹＭを培養し、ヒト細胞性フィブロネクチンを製造する方法について説明する。

【００２０】細胞株ＨＵＨ－６ＹＭの培養は、種培養としてシャーレで培養する。継代を繰り返し、次の連続培養で必要な細胞数を確保する。使用する培地としては、市販されているｅ－ＲＤＦ培地が適しているが、これに限定されるものではなく、その成分に準じて調製される培地はどのようなものでも使用可能である。また添加成分としては、タンパク性因子は特に必要ないが、細胞に悪影響を与えない限り、添加されていてもかまわない。

【００２１】培養条件は、特に規定されるものではないが、培養温度は３６～３７℃で、気相条件は、シャーレ等による種培養において５％程度の$CO_2$を含有する空気が適当である。しかし、連続培養においては特に$CO_2$等を調製した空気の必要はない。

【００２２】連続培養の方法は、特に限定されるものではないが、一般的な方法であるローラーボトルを用いた方法で行うことが可能である。この場合、そのローラーボトル内の細胞数に応じて培地の交換を適切な時期に行っていくものである。

【００２３】このような方法で得られた細胞株ＨＵＨ－６ＹＭの培養上清が、次の精製の原料となる。

【００２４】細胞性フィブロネクチンの精製には、一般的なタンパク質精製に用いられる方法を用いることができるが、その１例をあげれば次の通りである。

【００２５】まず培養上清に適当なプロテアーゼインヒビターを添加し、次に遠心分離に付し細胞片等の不純物を除去した後に、限外ロ過法によって濃縮を行い、培養上清中の細胞性フィブロネクチンを適当な濃度にまで上げる。これは次の精製を効率化するためである。濃縮された上清は、ＰＢＳ（－）（Ｐｈｏｓｐｈａｔｅ　Ｂｕｆｆｅｒｅｄ　Ｓａｌｉｎｅ）で平衡化したゼラチンアフィニティーカラムに負荷する。その後、ＰＢＳ（－）に６Ｍ尿素を溶解した緩衝液で溶出させ、その溶出画分を得る。この画分中での細胞性フィブロネクチンの純度は、電気泳動法による検定によると９５％以上であるが、さらに純度を高めるためには、一般的なイオン交換法などの他の方法を用いて高純度化することができるが、細胞性フィブロネクチンは、通常ＰＢＳ（－）のような緩衝液中では非常に溶解度が低いため、すべて尿素存在下で分離操作を行う必要がある。

【００２６】その後、尿素を除去し、ある濃度以上の細

胞性フィブロネクチン溶液を調製するために５％ショ糖を含むリン酸緩衝液と緩衝液交換を行う。緩衝液で交換を行う方法としては、一般的な方法である膜透析法や限外ロ過を用いてダイアフィルトレーション法によって行うことができる。

【００２７】このようにして得られるヒト細胞性フィブロネクチンは、二量体と単量体の混合物として得られる。細胞性フィブロネクチンの構造上の特徴としては、還元条件下及び非還元条件下のＳＤＳポリアクリルアミドゲル泳動の分析によると、その分子量が血漿性フィブロネクチン標品より大きく、また市販の抗細胞性フィブロネクチン抗体と反応する。

【００２８】以下に細胞株ＨＵＨ－６ＹＭを用いて製造したヒト細胞性フィブロネクチンの諸性質を示す。

①分子量

還元及び非還元状態におけるＳＤＳポリアクリルアミドゲル電気泳動により二量体が４７０ｋｄ，単量体が２４５ｋｄと２２５ｋｄを示し、二量体と単量体の比が２：１である。

②等電点：ｐＨ５～６

③免疫学的特異性

市販の抗細胞性フィブロネクチン抗体（シグマ社製、ＭＯＮＯＣＬＯＮＡＬ　ＡＮＴＩ－ＣＥＬＬＵＬＡＲ　ＦＩＢＲＯＮＥＣＴＩＮ，Ｐｒｏｄｕｃｔ　Ｎｏ．Ｆ６１４０）と反応し、抗ヒトフィブロネクチン抗体（宝酒造製、ｃｌｏｎｅ３０－８）とも反応する。

④プロテアーゼによる断片化の結果、本発明のヒト細胞性フィブロネクチンのプロテアーゼによる断片は、ヒト血漿性フィブロネクチンのプロテアーゼ断片と明らかに異なっていた。

また本発明で製造されたヒト細胞性フィブロネクチンは、既に報告されている細胞接着促進活性、ゼラチンビーズ凝集能及び細胞伸展活性などフィブロネクチンの生物学的作用を有する。

【００２９】以上のように細胞株ＨＵＨ－６ＹＭを用い、タンパク質無添加培地で、大量の細胞を連続培養することで、非常に有利にヒト細胞性フィブロネクチンを製造することができる。

【００３０】

【実施例】

【Ｉ．物質の生産】種培養として、細胞株ＨＵＨ－６ＹＭ（ＦＥＲＭ　Ｐ－１２８９０）をｅ－ＲＤＦ培地（極東製薬製）を用いて継代培養した。培養は３７℃、５％$CO_2$を含む空気で、１ヶ月間行った。

【００３１】培養上清を得るための連続培養は、１７５０ｃｍ$^2$のローラーボトル（ファルコン社製）１本当たり１×１０$^9$個の細胞を播種し、３７℃、大気下で培養した。播種後３週間までは、２～３日間隔で、その後は毎日ローラーボトル１本当たり５００ｍｌの培地を全量交換し、得られた培養上清を回収した。連続培養は約３



カ月行った。（図１）

【００３２】培養上清は、プロテアーゼインヒビターとして５ｍＭエチレンジアミン四酢酸ナトリウム（ＥＤＴＡ）を添加した後、連続遠心分離し１５００ｇ、流速２０Ｌ／分、（国産製Ｈ６００－Ｓ）により細胞断片等の不純物を除去し上澄を回収した。

【００３３】次に、分画分子量１００ｋ（フィルトロン社製、型式ＯＳ１００ＣＯ５）の限外ロ過膜を用いて先の上澄の１０倍濃縮液を調製した。これを、ＰＢＳ（－）で平衡化したゼラチンセファロース４Ｂカラム（ファルマシア社製）負荷し、ＰＢＳ（－）で洗浄し、平衡化した。続いてＰＢＳ（－）に６Ｍ尿素を溶解させた緩衝液で溶出を行った。

【００３４】この溶出液中の細胞性フィブロネクチンの純度は、還元状態下でのＳＤＳ－ポリアクリルアミドゲル電気泳動法による検定の結果９５％以上であった。

【００３５】この溶出液を集め、分画分子量１００ｋの限外ロ過膜（フィルトロン社製、型式ＯＳ－１００ＣＯ１）を用いて、まず溶出液を濃縮し、続いてダイアフィルトレーション法により、５％ショ糖を含む０．１Ｍリン酸ナトリウム緩衝液と溶媒交換を行った。

【００３６】以上のような方法で、ヒト細胞性フィブロネクチン標品が得られた。

【００３７】

【II. 物質の性質】

（１）分子量

【００３８】上記、ヒト細胞性フィブロネクチン標品の分子量をＳＤＳポリアクリルアミドゲル電気泳動法によって、標準タンパク質及びヒト血漿性フィブロネクチン標品（岩城硝子社製）と還元及び非還元状態下で比較した。

【００３９】その結果、本発明のヒト細胞性フィブロネクチン標品の分子量は二量体が４７０ｋｄ、単量体が２４５ｋｄと２２５ｋｄと推定され、ヒト血漿性フィブロネクチンよりも大きな分子量を有することが明らかになかった。（図２）

（２）免疫学的反応性

【００４０】血漿性フィブロネクチンには反応せず、細胞性フィブロネクチンのみに反応する抗細胞性フィブロネクチン（シグマ社製、Ｐｒｏｄｕｃｔ　Ｎｏ．Ｆ－６１４０）を用いてウエスタンブロッティング後の免疫染色法により反応性の確認を行った。その結果、本標品はこの特異抗体と反応した。またヒトフィブロネクチン抗体（宝酒造製、ｃｌｏｎｅ３０－８）とも反応した。

（３）プロテアーゼによる断片化

【００４１】サーモリシンを用いてヒト血漿性フィブロネクチンと本発明の細胞性フィブロネクチンのプロテアーゼによる断片の分子量の差について検討を行った。さらに得られた断片物の免疫学的反応性について検討した。

【００４２】その結果、本発明の細胞性フィブロネクチンはプロテアーゼによる断片の大きさがヒト血漿性フィブロネクチンとは明らかに異なり、さらのモノクローナル抗体による断片の免疫学的反応性によりＥＤ－Ａ及びＥＤ－Ｂ領域を含む細胞性フィブロネクチンであると考えられた。

【００４３】上記の分子量と免疫学的反応性、プロテアーゼによる断片及び細胞のｍＲＮＡの分析の結果、本発明で得られたフィブロネクチンは、ＥＤ－Ａ及びＥＤ－Ｂ領域を含むヒト細胞性フィブロネクチンであると判断された。

【００４４】（４）水溶性

既報によると、細胞性フィブロネクチンはｐＨ６から８の緩衝液に対し、非常に溶解性が低いことが報告されている。

【００４５】本発明においては、細胞性フィブロネクチンが、ショ糖を含む緩衝液に対し易溶性であることが見いだされた。各ショ糖濃度に対する溶解性は次の表１に示される。

【００４６】

【表１】

**表１．ショ糖溶液に対する溶解性**

| ショ糖濃度 | 透析後$OD_{280}$ | 初期液量 | 最終液量 | 回収率 |
|---|---|---|---|---|
| ０％ | 1.37 | ３mL | 3.0mL | 66％ |
| １％ | 1.53 | ４mL | 3.3mL | 60％ |
| ３％ | 1.93 | ３mL | 2.5mL | 77％ |
| ５％ | 2.45 | ３mL | 2.3mL | 90％ |
| 10％ | 3.78 | ３mL | 1.5mL | 90％ |

回収率＝(初期液量×透析後$OD_{280}$／1.3*)／(最終液量×2.08**／1.3)×100 (％)

*　細胞性フィブロネクチン濃度１mg／mlの時、$OD_{280}$＝1.3

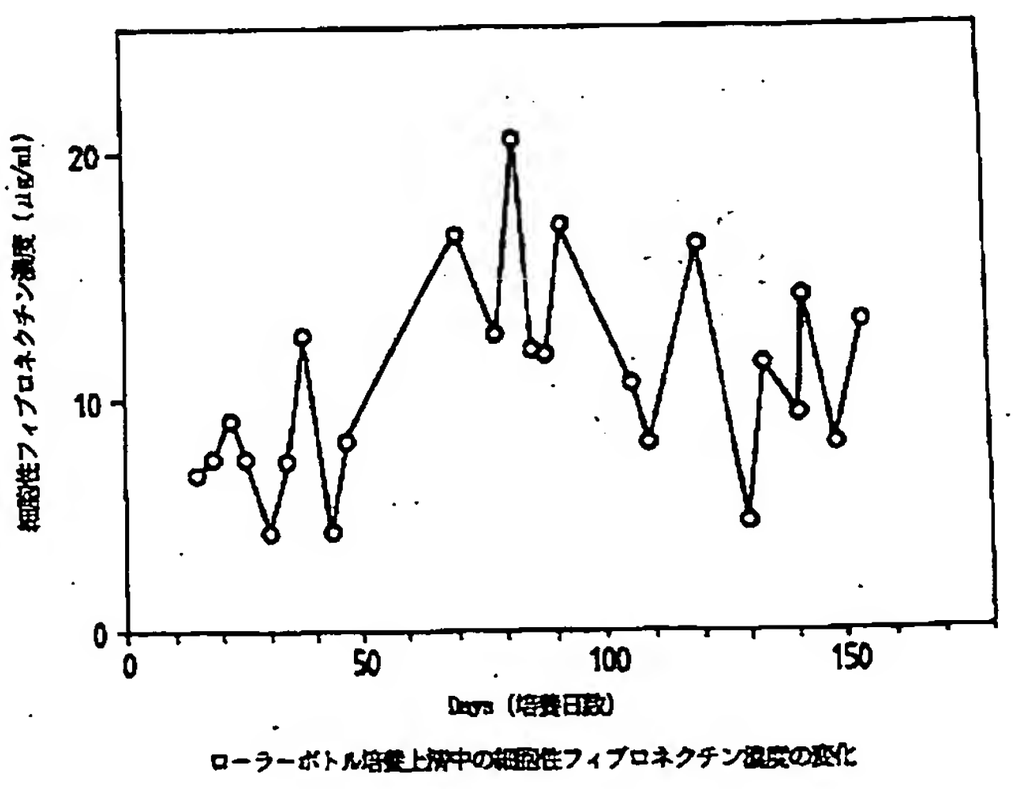
**　透析前サンプル溶液の$OD_{280}$

【図面の簡単な説明】

【図１】連続培養中において細胞株ＨＵＨ－６ＹＭが、培養上清中に産生するフィブロネクチン濃度の培養期間に対する変化を示す図。

【図２】本発明のヒト細胞性フィブロネクチンをＳＤＳポリアクリルアミドゲル電気泳動にかけた結果を示す。比較対照としては、ヒト血漿性フィブロネクチン標品を用いている。

【図１】




ローラーボトル培養上清中の細胞性フィブロネクチン濃度の変化

【図２】

図２の１

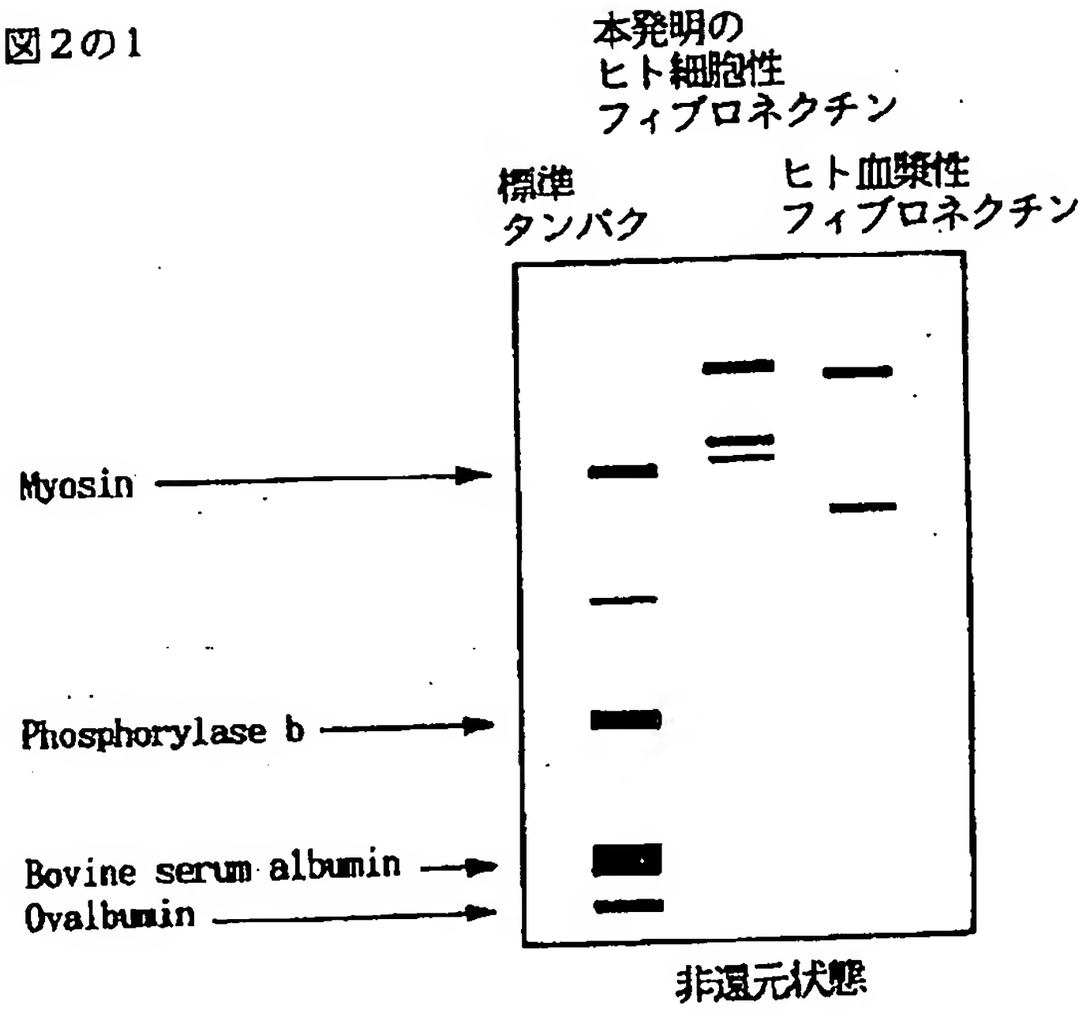
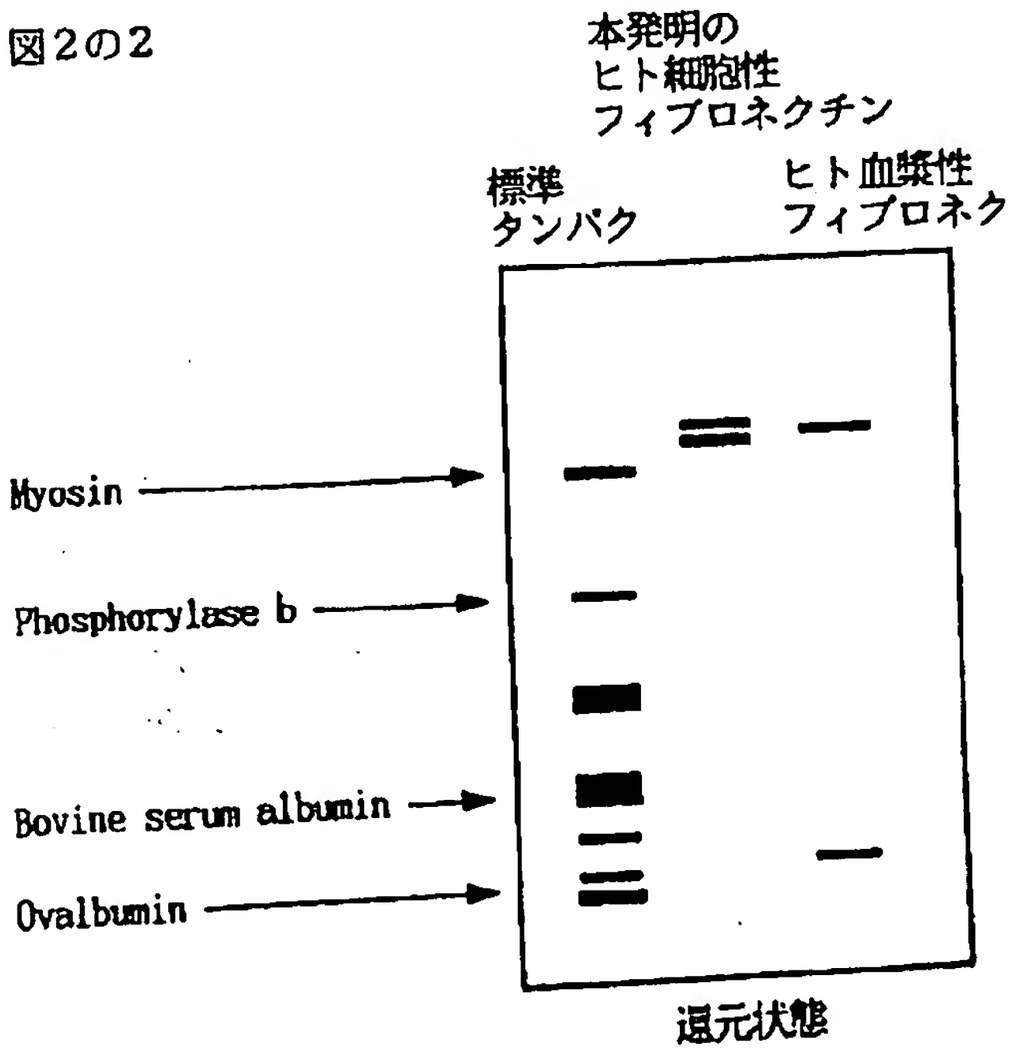

本発明の
ヒト細胞性
フィブロネクチン
標準
タンパク
ヒト血漿性
フィブロネクチン
Myosin
Phosphorylase b
Bovine serum albumin
Ovalbumin
非還元状態

図２の２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